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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/87, C07K 14/235, 14/245, 14/32, 14/34, 14/395, A61K 31/70

A1

(11) International Publication Number: **WO 98/59065**

(43) International Publication Date: 30 December 1998 (30.12.98)

(21) International Application Number: PCT/IB98/01005

(22) International Filing Date: 18 June 1998 (18.06.98)

(30) Priority Data:
9713122.1 20 June 1997 (20.06.97) GB

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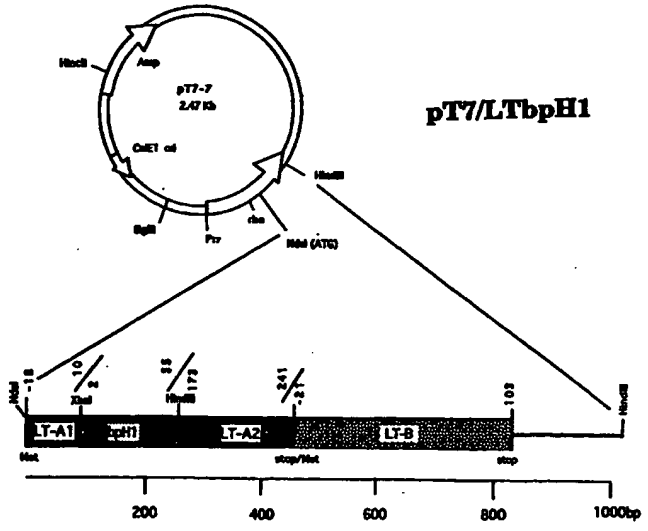
(81) Designated States: JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NOVEL METHOD OF DNA TRANSFER INTO CELLS



(57) Abstract

A novel system of DNA transfer into cells is described which utilises receptor–mediated endocytosis as the method of DNA entry. Disclosed are modified toxin molecules or toxin–like molecules which comprise DNA–binding motifs. These modified toxin or toxin–like molecules, which are capable of cellular internalisation by receptor–mediated endocytosis, are rendered non–toxic and can bind DNA to form modified toxin/toxin–like–DNA conjugates. The modified toxin/toxin–like–DNA conjugates, once cellularly internalised, are capable of releasing their "passenger DNA", thereby transforming or transfecting the cell. Also disclosed are methods of DNA transfer utilising the modified toxin or toxin–like molecules and uses of the same.

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NOVEL METHOD OF DNA TRANSFER INTO CELLS

The present invention relates to a novel method of facilitating DNA transfer into cells. The present invention more specifically relates to [DNA-binding]-[cell-binding] conjugates which are capable of binding to both DNA and cell surface receptors, thereby facilitating DNA uptake by cells by receptor-mediated endocytosis. Even more specifically, the present invention relates to toxins or toxin-like molecules which have been engineered chemically or by genetic engineering to become [DNA-binding]-[cell-binding] conjugates capable of mediating DNA transfer into cells.

BACKGROUND OF THE INVENTION

In order that genetic or chemical modifications can be made to cells, means have had to be devised to transfer the genetic material or chemicals necessary to accomplish the modification into the cells. Such transferred genetic material or chemicals include nucleic acids, peptides, polypeptides, proteins or other chemical or biochemical agents.

Numerous methods currently exist for facilitating DNA transfer into cells. Amongst the most commonly used are the methods of using competent cells (calcium chloride-treated bacterial cells), electroporation (prokaryotic and eukaryotic cells), calcium phosphate co-precipitation (mammalian cells), fusion of spheroplasts with polyethylene glycol (PEG) in the presence of DNA and calcium chloride (yeast cells), viral infection (prokaryotic and eukaryotic cells), and direct micro-injection (eukaryotic cells).

Bacterial transformation (usually with the genetic engineering workhorse, *Escherichia coli*) can be carried out

using bacterial cells rendered "competent" for DNA transfer (Cohen *et al.*, 1972). "Competent cells" are bacterial cells grown up to a particular cell density (log-phase), chilled, pelleted, resuspended in ice-cold calcium chloride (e.g. 50 mM) at a particular cell density (e.g. 10^{10} cells/ml). After about 30 min on ice, the now "competent" calcium chloride-treated cells can be either aliquotted, frozen on dry ice in the presence of glycerol and then transferred to -80°C for storage, or they can be used directly for transformation with DNA. Transformation protocols vary, but they usually involve incubating the competent cells on ice in the presence of the appropriate transforming DNA for about 30 min and then, depending on the protocol and cell type, subjecting the cells to a brief heat shock at 42°C. After the heat shock, the cells are usually transferred to a non-selective nutrient medium and incubated for about 30 min to 1 hr, after which the cells are transferred to a selective medium (usually agar plates incorporating appropriate chemical selection). Suitably transformed cells that have survived the transformation regime will be able to grow in the selective medium.

The common method of DNA electroporation (Zimmerman and Pheiffer, 1983; Neumann *et al.*, 1982; Potter *et al.*, 1984) utilises the physical effects of an locally generated short electrical impulse to disrupt the prokaryotic or eukaryotic cell membrane to create pores through which the DNA can enter the cell. Although the number of cells which do not survive this method of DNA transfer is fairly high, those that do are often successfully transformed or transfected.

The method of calcium phosphate co-precipitation (Graham and van der Eb, 1973) is well known and still commonly used to transfer DNA into mammalian cells. This method involves the uptake by the cells of calcium phosphate by endocytosis in the presence of DNA. Although the cell survival rate is often higher than with electroporation, the rate of successful DNA transfection is generally relatively low.

Viral infection can be used to transfer DNA from a virus (whether viral or foreign DNA) into an appropriate cell which is susceptible to infection (see, for example, Keown *et al.*, 1990).

Direct micro-injection (see Keown *et al.*, 1990) involves the direct insertion of DNA into a cell using physical means (i.e. via injection using an ultra-thin needle). Such techniques are time-consuming (because fewer cells can be transfected) but do achieve a high transfection rate.

However, as with all forms of DNA transfer into cells, the rate of transformation or transfection not only depends on the cell type and health, the method of transfer, and the conditions of culture, but also on the DNA itself (i.e. its size, conformation, strandedness, etc.).

Other methods of DNA transfer into cells also exist. Such methods include liposome fusion (lipofection; Deshayes *et al.*, 1985) and gold or tungsten microparticle bombardment of cells (biolistics; Klein *et al.*, 1987) in the presence of DNA.

Cells which are chemically modified (without actual genetic manipulation) are often those into which a selective killing agent is desired to be introduced. Thus, the targeting of cytotoxic or cytolytic chemicals such as anti-cancer agents into tumour cells can often be achieved by the selective transfer (by receptor-mediated endocytosis) of the toxic or lytic agent into the desired cell type.

The concept of receptor-mediated uptake of selected agents into cells has been recently used to simultaneously transfer DNA and agent into cells. Wagner, *et al.* (1990) have developed a transferrin-based receptor-mediated system for nucleic acid transfer into cells. This system involves covalently linking human transferrin or its chicken

homologue, conalbumin, to polycations such as the small DNA-binding protein protamine or to polylysines of various sizes via a disulphide bridge. These conjugated transferrin molecules maintain their ability to bind to their cognate receptors and mediate efficient iron transport into cells. The transferrin-polycation conjugates can form electrophoretically stable complexes with double-stranded DNA and modified RNA molecules. Once stably bound to nucleic acid, for instance DNA, the transferrin-polycation-DNA conjugate can bind to the transferrin receptor of a cell and be taken up into the cell by receptor-mediated endocytosis, thereby transferring the DNA into the cell where the ingested nucleic acid is released from the endosomal vesicles and can be expressed.

There can, however, be problems with the release of the ingested nucleic acid from the endosomal vesicles. The efficiency of such release has been noted to be increased by either repeated addition of the transferrin-polycation-nucleic acid conjugates or by addition of agents which affect endosomal and lysosomal pH (e.g. chloroquine). However, the repeated addition of the transferrin-polycation-nucleic acid conjugate is both time consuming and impractical and the addition of vesicle-affecting agents can be toxic to the treated cells.

As noted above, cells can be modified not only by the uptake of genetic material, but also by the uptake of chemical agents. Such chemical agents can either modify cells in some way or can even destroy or disable cells.

Toxins are chemical agents which are cytotoxic in nature and can potentially be used in modern medical treatments to selectively target and destroy improperly functioning/growing cells such as cancer cells (Pugsley, 1996). Such toxins often exert their toxic affects on cells once they have been taken up by toxin-sensitive cells (usually by receptor-mediated endocytosis).

The idea of selectively targeting toxins to particular cell types is known and toxin-ligand conjugates have been developed which bind to specific cell receptors (on certain cells). These cell-bound toxin-ligand conjugates are then endocytosed, thereby internally releasing the toxin to act upon the cell (Pugsley, 1996).

Using the principle of receptor-mediated uptake of toxins, a number of researchers have developed methods of delivering proteins into cells by fusing them with minimal toxin polypeptides. This allows the uptake of such fusion proteins simultaneously with the uptake of the toxin amino acid sequences which, through specific deletions, have been rendered non-toxic but still internalisable by receptor-mediated endocytosis.

The bacterial anthrax lethal toxin is a complex of protective antigen (PA), lethal factor (LF) and edema factor (EF). LF and EF bind by means of their amino-terminal domains to PA on the surface of toxin-sensitive cells and are translocated by receptor-mediated endocytosis into the cytosol, where they act on intracellular targets (Blanke *et al.*, 1996). Genetically fusing the amino-terminal domain of the lethal factor (LF) of anthrax toxin to certain heterologous proteins has been shown to potentiate these proteins for protective antigen (PA)-dependent delivery into mouse macrophage cells (Arora and Leppla, 1993). As a further development to this method, Blanke *et al.* (1996) found that short tracts of lysine, arginine, or histidine residues could be used in place of the amino-terminal sequences of LF and could also potentiate a protein (in this case, the enzymic A chain of diphtheria toxin (DTA)) for such PA-mediated transfer into cells.

The use of antibodies in connection with the transfer of DNA into cells has also been investigated. Fominaya and Wels (1996) have constructed a recombinant fusion protein

which serves as a target cell-specific carrier for the transfer of DNA via receptor-mediated endocytosis. The recombinant protein consists of three functional domains: (1) an ErbB-2-specific single chain antibody which confers target cell specificity, (2) the translocation domain of the bacterial *Pseudomonas* exotoxin A which facilitates endosome escape of the DNA, and (3) a DNA-binding domain derived from the yeast GAL4 protein which enables sequence-specific binding to DNA. Complexes of this fusion protein bound to plasmid DNA carrying a reporter gene and a GAL4-specific recognition sequence, after condensation and charge-neutralisation with poly-L-lysine, has been shown to transfect ErbB-2-expressing tumour cells *in vitro* in cell-specific manner.

However, the direct use of toxin or toxin-like amino acid sequences linked to DNA-binding motifs for the mediation of DNA transfer into cells has not been previously disclosed.

It has been found that modified toxin or toxin-like amino acid sequences, which are capable of participating in receptor-mediated endocytosis but which are non-toxic, can be used to transfer DNA into cells. The toxin or toxin-like sequences are modified by the chemical or genetically-engineered addition of DNA-binding motifs thereto to produce modified toxin or toxin-like molecules capable of binding DNA molecules. DNA, once bound to the modified toxin or toxin-like molecule, results in a modified toxin/toxin-like-DNA conjugate which is capable of binding to toxin molecule-sensitive or toxin-like molecule-sensitive cells (i.e. those cells which possess receptors which are recognised by the toxin or toxin-like molecules or those cells for which semi-specific targeting is possible). The DNA can then be internalised into the cells through receptor-mediated endocytosis of the modified toxin/toxin-like-DNA conjugate. Once inside the cells, the DNA is released, making it capable of transfecting the cells.

SUMMARY OF INVENTION

According to a first aspect of the present invention there is provided a modified toxin molecule or toxin-like molecule comprising:

(i) one or more toxin or toxin-like amino acid sequences capable of binding to appropriate receptors on a cell surface and being internalised into the cell by receptor-mediated endocytosis, and

(ii) one or more DNA-binding motifs;

wherein the toxin or toxin-like amino acid sequences are incapable of effecting toxicity or only effect low residual toxicity.

Preferably, the toxin amino acid sequences of the modified toxin molecule are derived from one or more bacterial toxins. More preferably, the toxin sequences are derived from one or more bacterial toxins of *Pseudomonas* sp., preferably *Pseudomonas aeruginosa*, *Corynebacterium diphtheriae*, *Bordetella pertussis*, *Vibrio cholerae*, *Clostridium* sp., preferably *Clostridium botulinum*, or *Escherichia coli*.

Preferably, the toxin-like amino acid sequences of the modified toxin-like molecule are derived from one or more ADP-ribosylating enzymes. More preferably, the toxin-like sequences are derived from ADP-ribosylating enzymes such as eukaryotic mono-ADP-ribosyltransferases (MARTs), T-cell alloantigens, poly-ADP-ribosylpolymerases (PARPs), the bacterial metabolism regulation factor, DraT, or enzymes encoded by T-even bacteriophages.

The preferred T-cell alloantigens are RT6, RT6-1, or RT6-2 and the preferred T-even bacteriophages are T2, T4, or T6.

The DNA-binding motif(s) may comprise all or part of one or more DNA-binding proteins. Such DNA-binding proteins can be selected from, for example, those proteins with limited specificity for DNA sequence, such as histone or histone-like DNA-binding proteins or polylysine, those proteins showing DNA sequence specificity such as the yeast transcriptional activator GAL4, and activators and repressors of transcription which selectively bind specific nucleotide motifs. Preferably, the DNA-binding motif(s) comprise(s) all or part of a histone or histone-like protein, optionally BpH1, or the yeast transcriptional activator GAL4.

The modified toxin or toxin-like molecule according to the preceding aspect can be produced chemically or by genetic engineering. Such chemical or genetic engineering methods of production can include chemical alteration of the amino acid sequences of the molecule or alteration of the molecule at the DNA level using molecular biological methods well known in the art. Using the techniques well known in the field of genetic engineering, the DNA encoding the amino acid sequence(s) of the toxin or toxin-like molecule can be deleted, added to, mutated, or substituted in order to establish the desired modification of the expressed molecule.

According to a second aspect of the present invention there is provided a modified toxin or toxin-like molecule as described above, wherein the modified toxin or toxin-like molecule is further modified to make it capable of binding to appropriate receptors other than receptors specific for all or part of the toxin or toxin-like amino acid sequences of the molecule.

According to a third aspect of the present invention there is provided a modified toxin or toxin-like molecule as described in any of the preceding aspects, further

comprising DNA bound to the DNA-binding motif(s).

Preferably, the modified toxin or toxin-like molecule according to the third aspect of the present invention has bound to it DNA which (i) encodes one or more bacterial, viral, fungal or parasitic proteins, cytotoxic agents, cytolytic agents, antigens, antigenic epitopes, antibodies or fragments thereof, or (ii) comprises one or more replacement genes, augmentative genes or additional genes. More preferably, the DNA bound to the modified toxin or toxin-like molecule can encode antigens or antigenic epitopes which are antigens or antigenic epitopes of a pathogen or, even more preferably, protective antigens or protective epitopes of a pathogen.

According to a fourth aspect of the present invention there is provided a pharmaceutical composition comprising a modified toxin or toxin-like molecule as described in any of the preceding aspects and a pharmaceutically acceptable excipient.

According to a fifth aspect of the present invention there is provided a process for the production of a modified toxin or toxin-like molecule according to any of the preceding aspects comprising modifying the toxin or toxin-like amino acid sequences by:

(i) eliminating or reducing toxicity, if the amino acid sequences are toxic, and

(ii) incorporating one or more DNA-binding motifs into the molecule.

Reducing toxicity rather than completely eliminating toxicity may be preferable to obtain adjuvanticity.

Preferably, in the process according to the fifth aspect of the present invention, the modification for eliminating or

reducing toxicity includes addition, deletion, or substitution of amino acids.

Preferably, in the process according to the fifth aspect of the present invention, the modification for incorporating the DNA-binding motif(s) is addition of all or part of one or more DNA-binding proteins. Such DNA-binding proteins can be selected from, for example, those proteins with limited specificity for DNA sequence, such as histone or histone-like DNA-binding proteins or polylysine, those proteins showing DNA sequence specificity such as the yeast transcriptional activator GAL4, and activators and repressors of transcription which selectively bind specific nucleotide motifs. The preferred DNA-binding protein is a histone or histone-like protein, optionally BpH1, or the yeast transcriptional activator GAL4. In the preferred modification for incorporating the DNA-binding motif(s), the addition of all or part of one or more DNA-binding proteins is via one or more chemical or genetically engineered linkage moieties.

According to a sixth aspect of the present invention there is provided a process for the production of a pharmaceutical composition according to the fourth aspect of the present invention, comprising:

(i) modifying the toxin or toxin-like amino acid sequences according to the process of any of the preceding aspects, and

(ii) combining the modified toxin or toxin-like molecule of step (i) with a pharmaceutically acceptable excipient.

According to a seventh aspect of the present invention there is provided a method of DNA transfer into cells comprising:

(i) binding DNA desired to be transferred into cells to the modified toxin or toxin-like molecule according to the

first aspect of the present invention via one or more of the DNA-binding motifs to form a modified toxin/toxin-like-DNA conjugate,

(ii) incubating the modified toxin/toxin-like-DNA conjugate with appropriate receptor-bearing cells, thereby allowing the conjugate to bind to the cells via the receptors,

(iii) selecting or screening for cells which have been transformed or transfected with the DNA, wherein the DNA has been cellularly internalised via receptor-mediated endocytosis.

According to an eighth aspect of the present invention there is provided a use of the modified toxin or toxin-like molecule according to any of the first, second or third aspects of the present invention in the *in vitro* or *in vivo* transfer of DNA into cells.

Preferably, in the use according to the eighth aspect of the present invention, the DNA to be transferred into cells is bound to one or more of the DNA-binding motifs of the modified toxin or toxin-like molecule, resulting in a modified toxin/toxin-like-DNA conjugate which is then internalised into the cells by receptor-mediated endocytosis.

According to a ninth aspect of the present invention there is provided a use of the modified toxin or toxin-like molecule according to any of the first, second or third aspects of the present invention in the *in vitro* or *in vivo* transformation or transfection of cells.

According to a tenth aspect of the present invention there is provided a use of the modified toxin or toxin-like molecule according to any of the first, second or third aspects of the present invention as a pharmaceutical.

According to an eleventh aspect of the present invention there is provided a use of the modified toxin or toxin-like molecule according to any of the first, second or third aspects of the present invention or the pharmaceutical composition of the fourth aspect of the present invention, in the manufacture of a medicament for:

(i) vaccination;

(ii) gene therapy;

(iii) treating or destroying diseased cells.

Preferably, in the use according to the eleventh aspect of the present invention, the vaccination can involve activation of the cytotoxic T-cell immune response or humoral immune response.

Preferably, in the use according to the eleventh aspect of the present invention, the gene therapy can include the replacement, augmentation, or addition of genes.

Preferably, in the use according to the eleventh aspect of the present invention, the diseased cells can include cancer cells, microbially-infected cells, or abnormal cells.

According to a twelfth aspect of the present invention there is provided a kit for the *in vitro* or *in vivo* transfer of DNA into cells, comprising the modified toxin or toxin-like molecule according to any of the first, second or third aspects of the present invention or the pharmaceutical composition of the fourth aspect of the present invention.

According to a thirteenth aspect of the present invention there is provided a kit for the *in vitro* or *in vivo* transformation or transfection of cells, comprising the modified toxin or toxin-like molecule according to any of the first, second or third aspects of the present invention

or the pharmaceutical composition of the fourth aspect of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature (see, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, 1989; D.N Glover (ed.), *DNA Cloning, Volumes I and II*, 1985; M.J. Gait (ed.), *Oligonucleotide Synthesis*, 1984; B.D. Hames and S.J. Higgins (eds.), *Nucleic Acid Hybridization*, 1984; B.D. Hames and S.J. Higgins (eds.), *Transcription and Translation*, 1984; R.I. Freshney (ed.), *Animal Cell Culture*, 1986; *Immobilized Cells and Enzymes*, IRL Press, 1986; B. Perbal, *A Practical Guide to Molecular Cloning*, 1984; The series, *Methods in Enzymology*, Academic Press, Inc.; J.H. Miller and M.P. Calos (eds.), *Gene Transfer Vectors for Mammalian Cells*, Cold Spring Harbor Laboratory, 1987; Wu and Grossman (eds.) and Wu (ed.), *Methods in Enzymology, Volumes 154 and 155*, respectively; Mayer and Walker (eds.), *Immunochemical Methods in Cell and Molecular Biology*, Academic Press, London, 1987; Scopes, *Protein Purification: Principles and Practice*, Second Edition, Springer-Verlag, New York, 1987; and D.M. Weir and C. C. Blackwell (eds.), *Handbook of Experimental Immunology, Volumes I-IV*, 1986).

A significant advantage of producing heterologous proteins by recombinant DNA techniques rather than by isolating and purifying a protein from natural sources is that equivalent quantities of the protein can be produced by using less starting material than would be required for isolating the protein from a natural source. Producing the protein by recombinant techniques also permits the protein to be

isolated in the absence of some molecules normally present in cells. Indeed, protein compositions entirely free of any trace of human protein contaminants can readily be produced because the only human protein produced by the recombinant non-human host is the recombinant protein at issue. Potential viral agents from natural sources and viral components pathogenic to humans are also avoided.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes) and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents (adjuvants).

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: aluminum salts (alum) such as aluminium hydroxide, aluminium phosphate, aluminium sulphate etc., oil emulsion formulations, with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components, such as for example (1) MF59 (Published International patent application WO-A-90/14837, containing 5% Squalene, 0.5% Tween® 80, 0.5% Span® 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA 02164, USA), (2) SAF, containing 10% squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (3) RIBI™ adjuvant system (RAS) (Ribi Immunochem, Hamilton, MT, USA) containing 2% Squalene, 0.2% Tween® 80 and one or

more bacterial cell wall components from the group consisting of monophosphoryl lipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS) preferably MPL+CWS (Detox™), muramyl peptides such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE) etc., and cytokines, such as interleukins (IL-1, IL-2 etc.), macrophage colony stimulating factor (M-CSF), tumour necrosis factor (TNF) etc. Additionally, saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA, USA) may be used or particles generated therefrom such as ISCOMS (immunostimulating complexes). Furthermore, Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA) may be used. Alum and MF59 are preferred.

The immunogenic compositions (e.g. the antigen, pharmaceutically acceptable carrier and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a

series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., nonhuman primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally, e.g. by injection either subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analogue, internucleotide modifications

such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins (including, for example, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

A "replicon" is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc. that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control. This may include selectable markers.

A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is translated into a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, cDNA, and recombinant polynucleotide sequences.

"PCR" refers to the technique of polymerase chain reaction as described in Saiki *et al.*, *Nature, 324*: 163, 1986; Scharf *et al.*, *Science, 233*: 1076-1078, 1986; U.S. Patent 4,683,195; and U.S. Patent 4,683,202.

As used herein, x is "heterologous" with respect to y if x is not naturally associated with y in the identical manner; i.e., x is not associated with y in nature or x is not associated with y in the same manner as is found in nature.

"Homology" refers to the degree of similarity between x and y. The correspondence between the sequence from one form to another can be determined by techniques known in the art. For example, they can be determined by a direct comparison of the sequence information of the polynucleotide. Alternatively, homology can be determined by hybridization

of the polynucleotides under conditions which form stable duplexes between homologous regions (for example, those which would be used prior to S_1 digestion), followed by digestion with single-stranded specific nuclease(s), followed by size determination of the digested fragments.

As used herein, the term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

A polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.

The protein may be used for producing antibodies, either monoclonal or polyclonal, specific to the protein. The methods for producing these antibodies are known in the art.

"Recombinant host cells", "host cells", "cells," "cell cultures", and other such terms denote, for example, microorganisms, insect cells, and mammalian cells, that can

be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. Examples for mammalian host cells include Chinese hamster ovary (CHO) and monkey kidney (COS) cells.

Specifically, as used herein, "cell line" refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants. The term "cell line" also includes immortalized cells. Preferably, cell lines include nonhybrid cell lines or hybridomas to only two cell types.

As used herein, the term "microorganism" includes prokaryotic and eukaryotic microbial species such as bacteria and fungi, the latter including yeast and filamentous fungi.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

By "genomic" is meant a collection or library of DNA molecules which are derived from restriction fragments that

have been cloned in vectors. This may include all or part of the genetic material of an organism.

By "cDNA" is meant a complementary DNA sequence that hybridizes to a complementary strand of DNA.

By "purified" and "isolated" is meant, when referring to a polypeptide or nucleotide sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. The term "purified" as used herein preferably means at least 75% by weight, more preferably at least 85% by weight, more preferably still at least 95% by weight, and most preferably at least 98% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000, can be present).

Once the appropriate coding sequence is isolated, it can be expressed in a variety of different expression systems; for example those used with mammalian cells, baculoviruses, bacteria, and yeast.

i. Mammalian Systems

Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA

box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation (Sambrook *et al.*, "Expression of Cloned Genes in Mammalian Cells", in: *Molecular Cloning: A Laboratory Manual*, 2nd ed., 1989).

Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumour virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallothionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible), depending on the promoter can be induced with glucocorticoid in hormone-responsive cells.

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter (Maniatis *et al.*, *Science, 236*: 1237, 1987; Alberts *et al.*, *Molecular Biology of the Cell*, 2nd ed., 1989). Enhancer elements derived from viruses may be particularly useful, because they usually have a broader host range. Examples include the SV40 early gene enhancer [Dijkema *et al.*, *EMBO J.*, *4*: 761, 1985) and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus (Gorman *et al.*, *Proc. Natl. Acad. Sci. USA, 79*: 6777, 1982b) and from human cytomegalovirus (Boshart *et al.*, *Cell, 41*: 521, 1985). Additionally, some enhancers are regulatable and become

active only in the presence of an inducer, such as a hormone or metal ion (Sassone-Corsi and Borelli, *Trends Genet., 2*: 215, 1986; Maniatis *et al.*, *Science, 236*: 1237, 1987).

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus triparite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation (Birnstiel *et al.*, *Cell, 41*: 349, 1985; Proudfoot and Whitelaw, "Termination and 3' end processing of eukaryotic RNA", in: *Transcription and Splicing* (eds. B.D. Hames and D.M. Glover), 1988; Proudfoot, *Trends Biochem. Sci., 14*: 105, 1989). These sequences direct the transcription of an mRNA which can be translated into the

polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40 (Sambrook *et al* ., "Expression of cloned genes in cultured mammalian cells", in: *Molecular Cloning: A Laboratory Manual*, 1989).

Some genes may be expressed more efficiently when introns (also called intervening sequences) are present. Several cDNAs, however, have been efficiently expressed from vectors that lack splicing signals (also called splice donor and acceptor sites) (see, for example, Gothing and Sambrook, *Nature, 293*: 620, 1981). Introns are intervening noncoding sequences within a coding sequence that contain splice donor and acceptor sites. They are removed by a process called "splicing," following polyadenylation of the primary transcript (Nevins, *Ann. Rev. Biochem., 52*: 441, 1983; Green, *Ann. Rev. Genet., 20*: 671, 1986; Padgett *et al.*, Ann. Rev. Biochem. 55: 1119, 1986; Krainer and Maniatis, "RNA splicing", in: *Transcription and Splicing* (eds. B.D. Hames and D.M. Glover), 1988).

Usually, the above-described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 (Gluzman, *Cell, 23*: 175, 1981) or polyomaviruses, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr

virus. Additionally, the replicon may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a prokaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 (Kaufman *et al.*, *Mol. Cell. Biol.*, *9*: 946, 1989) and pHEBO (Shimizu *et al.*, *Mol. Cell. Biol.*, *6*: 1074, 1986).

The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), and a number of other cell lines.

ii. Baculovirus Systems

The polynucleotide encoding the protein can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques which are known in the art.

Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type

baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene into the baculovirus genome); and appropriate insect host cells and growth media.

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild-type viral genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego, CA, USA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555*, 1987 (hereinafter "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above-described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are usually assembled into an intermediate transplacement construct (transfer vector). This construct may contain a single gene and operably linked regulatory elements; multiple genes, each with its owned set of operably linked regulatory elements; or multiple genes, regulated by the same set of regulatory elements. Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as a bacterium. The replicon will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for

introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; see Luckow and Summers, *Virology, 17*: 31, 1989).

The plasmid usually also contains the polyhedrin polyadenylation signal (Miller *et al.*, *Ann. Rev. Microbiol.*, *42*: 177, 1988) and a prokaryotic ampicillin-resistance (amp) gene and origin of replication for selection and propagation in *Escherichia coli*.

Baculovirus transfer vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable of binding a baculovirus RNA polymerase and initiating the downstream (5' to 3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein (Friesen *et al.*, "The Regulation of Baculovirus Gene Expression", in: *The Molecular Biology of Baculoviruses* (ed. Walter Doerfler), 1986; and EPO Publ. Nos. 127 839 and 155 476) and the gene encoding the p10 protein (Vlak *et al.*, *J. Gen. Virol.*, *69*: 765, 1988).

DNA encoding suitable signal sequences can be derived from

genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell *et al.*, *Gene, 73*: 409, 1988). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human α-interferon (Maeda *et al.*, *Nature, 315*: 592, 1985), human gastrin-releasing peptide (Lebacq-Verheyden *et al.*, *Mol. Cell. Biol., 8*: 3129, 1988), human IL-2, Smith *et al.*, *Proc. Natl. Acad. Sci. USA, 82*: 8404, 1985), mouse IL-3 (Miyajima *et al.*, *Gene, 58*: 273, 1987) and human glucocerebrosidase (Martin *et al.*, *DNA, 7*: 99, 1988), can also be used to provide for secretion in insects.

A recombinant polypeptide or polyprotein may be expressed intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of nonfused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by *in vitro* incubation with cyanogen bromide.

Alternatively, recombinant polyproteins or proteins which are not naturally secreted can be secreted from the insect cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the translocation of the protein into the endoplasmic reticulum.

After insertion of the DNA sequence and/or the gene encoding the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild-type baculovirus - usually by co-transfection. The promoter and transcription termination sequence of the construct will usually comprise a 2-5kb section of the baculovirus genome. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art (see Summers and Smith, *supra*; Smith *et al.*, *Mol. Cell. Biol.*, *3*: 2156, 1983; and Luckow and Summers, *supra*). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene (Miller *et al.*, *Bioessays*, *4*: 91, 1989). The DNA sequence, when cloned in place of the polyhedrin gene in the expression vector, is flanked both 5' and 3' by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Homologous recombination occurs at low frequency (between about 1% and about 5%); thus, the majority of the virus produced after co-transfection is still wild-type virus. Therefore, a method is necessary to identify recombinant viruses. An advantage of the expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein, which is produced by the native virus, is produced at very high levels in the nuclei of infected cells at late times after viral infection. Accumulated polyhedrin protein forms occlusion bodies that also contain embedded particles. These occlusion bodies, up to 15 μm in size, are highly refractile, giving them a bright shiny appearance that is readily visualized under the light microscope. Cells infected with recombinant viruses lack occlusion bodies.

To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells by techniques known to those skilled in the art. Namely, the plaques are screened under the light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies (Ansubel *et al.* (eds.), *"Current Protocols in Microbiology", Vol. 2 at 16.8 (Supp. 10)*, 1990; Summers and Smith, *supra*; Miller *et al.*, *supra*).

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni* (PCT Pub. No. WO 89/046699; Carbonell *et al.*, *J. Virol.*, *56*: 153, 1985; Wright, *Nature*, *321*: 718, 1986; Smith *et al.*, *Mol. Cell. Biol.*, *3*: 2156, 1983; and see generally, Fraser, *et al.*, *In Vitro Cell. Dev. Biol.*, *25*: 225, 1989).

Cells and cell culture media are commercially available for both direct and fusion expression of heterologous polypeptides in a baculovirus/expression system; cell culture technology is generally known to those skilled in the art (see, e.g., Summers and Smith, *supra*).

The modified insect cells may then be grown in an appropriate nutrient medium, which allows for stable maintenance of the plasmid(s) present in the modified insect host. Where the expression product gene is under inducible control, the host may be grown to high density, and expression induced. Alternatively, where expression is constitutive, the product will be continuously expressed into the medium and the nutrient medium must be continuously circulated, while removing the product of interest and augmenting depleted nutrients. The product may be purified by such techniques as chromatography, e.g., HPLC, affinity

chromatography, ion exchange chromatography, etc.; electrophoresis; density gradient centrifugation; solvent extraction, or the like. As appropriate, the product may be further purified, as required, so as to remove substantially any insect proteins which are also secreted in the medium or result from lysis of insect cells, so as to provide a product which is at least substantially free of host debris, e.g., proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host cells derived from the transformants are incubated under conditions which allow expression of the recombinant protein encoding sequence. These conditions will vary, dependent upon the host cell selected. However, the conditions are readily ascertainable to those of ordinary skill in the art, based upon what is known in the art.

iii. Bacterial Systems

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3") transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if

present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the *lac* operon in *E. coli* (Raibaud *et al.*, *Ann. Rev. Genet., 18*: 173, 1984). Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) (Chang *et al.*, *Nature, 198*: 1056, 1977), and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*) (Goeddel *et al.*, *Nuc. Acids Res., 8*: 4057, 1980; Yelverton *et al.*, *Nuc. Acids Res., 9*: 731, 1981; U.S. Patent No. 4,738,921; and EPO Publ. Nos. 036 776 and 121 775). The g-laotamase (*bla*) promoter system (Weissmann, "The cloning of interferon and other mistakes", in: *Interferon 3* (ed. I. Gresser), 1981), and bacteriophage lambda PL (Shimatake *et al.*, *Nature, 292*: 128, 1981) and T5 (U.S. Patent No. 4,689,406) promoter systems also provide useful promoter sequences.

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter (U.S. Patent No. 4,551,433). For example, the *tac* promoter is a hybrid *trp-lac* promoter comprised of both *trp* promoter and *lac* operon sequences that is regulated by the *lac* repressor (Amann *et al.*, *Gene, 25*: 167, 1983; de Boer *et al.*, *Proc. Natl. Acad. Sci. USA, 80*: 21, 1983). Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A

naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled promoter system (Studier *et al.*, *J. Mol. Biol.*, *189*: 113, 1986; Tabor *et al.*, *Proc. Natl. Acad. Sci. USA*, *82*: 1074, 1985). In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an *E. coli* operator region (EPO Publ. No. 267 851).

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In *E. coli*, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon (Shine *et al.*, *Nature*, *254*: 34, 1975). The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' and of *E. coli* 16S rRNA (Steitz *et al.*, "Genetic signals and nucleotide sequences in messenger RNA", in: *Biological Regulation and Development: Gene Expression* (ed. R.F. Goldberger), 1979). To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site (Sambrook *et al.*, "Expression of cloned genes in *Escherichia coli*", in: *Molecular Cloning: A Laboratory Manual*, 1989).

A DNA molecule may be expressed intracellularly. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide or by either *in vivo* on *in vitro* incubation with a bacterial methionine N-terminal peptidase (EPO Publ. No. 219 237).

Fusion proteins provide an alternative to direct expression.

Usually, a DNA sequence encoding the N-terminal portion of an endogenous bacterial protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the bacteriophage lambda cell gene can be linked at the 5' terminus of a foreign gene and expressed in bacteria. The resulting fusion protein preferably retains a site for a processing enzyme (factor Xa) to cleave the bacteriophage protein from the foreign gene (Nagai *et al.*, *Nature, 309*: 810, 1984). Fusion proteins can also be made with sequences from the *lacZ* (Jia *et al.*, *Gene, 60*: 197, 1987), *trpE* (Allen *et al.*, *J. Biotechnol., 5*: 93, 1987; Makoff *et al.*, *J. Gen. Microbiol., 135*: 11, 1989), and *Chey* (EPO Publ. No. 324 647) genes. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated (Miller *et al.*, *Bio/Technology 7*: 698, 1989).

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein in bacteria (U.S. Patent No. 4,336,336). The signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites, which can be cleaved either *in vivo* or *in vitro* encoded between the signal peptide fragment and the foreign gene.

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the *E. coli* outer membrane protein gene (*ompA*) (Masui *et al.*, in: *Experimental Manipulation of Gene Expression*, 1983; Ghrayeb *et al.*, *EMBO J.*, *3*: 2437, 1984) and the *E. coli* alkaline phosphatase signal sequence (*phoA*) (Oka *et al.*, *Proc. Natl. Acad. Sci. USA*, *82*: 7212, 1985). As an additional example, the signal sequence of the alpha-amylase gene from various *Bacillus* strains can be used to secrete heterologous proteins from *B. subtilis* (Palva *et al.*, *Proc. Natl. Acad. Sci. USA*, *79*: 5582, 1982; EPO Publ. No. 244 042).

Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong promoters, such as the *trp* gene in *E. coli* as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a prokaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host

containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various *Bacillus* strains integrate into the *Bacillus* chromosome (EPO Publ. No. 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline (Davies *et al.*, *Ann. Rev. Microbiol.*, *32*: 469, 1978). Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extra-chromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For

example, expression vectors have been developed for, *inter alia*, the following bacteria: *B. subtilis* (Palva *et al.*, *Proc. Natl. Acad. Sci. USA, 79*: 5582, 1982; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541), *E. coli* (Shimatake *et al.*, *Nature, 292*: 128, 1981; Amann *et al.*, *Gene, 40*: 183, 1985; Studier *et al.*, *J. Mol. Biol., 189*: 113, 1986; EPO Publ. Nos. 036 776, 136 829 and 136 907), *Streptococcus cremoris* (Powell *et al.*, *Appl. Environ. Microbiol., 54*: 655, 1988); *Streptococcus lividans* (Powell *et al.*, *Appl. Environ. Microbiol., 54*: 655, 1988), and *Streptomyces lividans* (U.S. Patent No. 4,745,056).

Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the transformation of bacteria treated with $CaCl_2$ or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation. Transformation procedures usually vary with the bacterial species to be transformed (see, e.g., Masson *et al.*, *FEMS Microbiol. Lett., 60*: 273, 1989; Palva *et al.*, *Proc. Natl. Acad. Sci. USA, 79*: 5582, 1982; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541 [*Bacillus*], Miller *et al.*, *Proc. Natl. Acad. Sci. USA, 8*: 856, 1988; Wang *et al.*, *J. Bacteriol., 172*: 949, 1990 [*Campylobacter*], Cohen *et al.*, *Proc. Natl. Acad. Sci. USA, 69*: 2110, 1973; Dower *et al.*, *Nuc. Acids Res., 16*: 6127, 1988; Kushner, "An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids", in: *Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering* (eds. H.W. Boyer and S. Nicosia), 1978; Mandel *et al.*, *J. Mol. Biol., 53*: 159, 1970; Taketo, *Biochim. Biophys. Acta, 949*: 318, 1988 [*Escherichia*], Chassy *et al.*, *FEMS Microbiol. Lett., 44*: 173, 1987 [*Lactobacillus*], Fiedler *et al.*, *Anal. Biochem, 170*: 38, 1988 [*Pseudomonas*], Augustin *et al.*, *FEMS Microbiol. Lett., 66*: 203, 1990 [*Staphylococcus*], Barany *et al.*, *J. Bacteriol., 144*: 698, 1980; Harlander, "Transformation of *Streptococcus lactis* by electroporation", in: *Streptococcal Genetics* (ed. J. Ferretti and R. Curtiss

III), 1987; Perry *et al.*, *Infec. Immun.*, *32*: 1295, 1981; Powell *et al.*, *Appl. Environ. Microbiol.* *54*: 655, 1988; Somkuti *et al.*, *Proc. 4th Eur. Cong. Biotechnology*, *1*: 412, 1987 [*Streptococcus*]).

iv. Yeast Expression

Yeast expression systems are also known to one of ordinary skill in the art. A yeast promoter is any DNA sequence capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which, if present, is usually distal to the structural gene. The UAS permits regulated (inducible) expression. Constitutive expression occurs in the absence of a UAS. Regulated expression may be either positive or negative, thereby either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter sequences. Examples include alcohol dehydrogenase (ADH) (EPO Publ. No. 284 044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EPO Publ. No. 329 203). The yeast PHO5 gene, encoding acid phosphatase, also provides useful promoter sequences (Myanohara *et al.*, *Proc. Natl. Acad. Sci. USA*, *80*: 1, 1983).

In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Patent Nos. 4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the ADH2, GAL4, GAL10, or PHO5 genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (EPO Publ. No. 164 556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription. Examples of such promoters include, *inter alia*, Cohen *et al.*, *Proc. Natl. Acad. Sci. USA*, *77*: 1078, 1980; Henikoff *et al.*, *Nature*, *283*: 835, 1981; Hollenberg *et al.*, *Curr. Topics Microbiol. Immunol.*, *96*: 119, 1981; Hollenberg *et al.*, "The Expression of Bacterial Antibiotic Resistance Genes in the Yeast *Saccharomyces cerevisiae*", in: *Plasmids of Medical, Environmental and Commercial Importance* (eds. K.N. Timmis and A. Puhler), 1979; Mercerau-Puigalon *et al.*, *Gene*, *11*: 163, 1980; Panthier *et al.*, *Curr. Genet.*, *2*: 109, 1980.

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other

stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site (see, e.g., EPO Publ. No. 196 056). Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can be isolated (see, e.g., PCT Publ. No. WO 88/024066).

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provide for secretion in yeast of the foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene (EPO Publ. No. 012 873; JPO Publ. No. 62,096,086) and the A-factor gene (U.S. Patent No. 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (EPO Publ. No. 060 057).

A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the

full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (usually about 25 to about 50 amino acid residues) (U.S. Patent Nos. 4,546,083 and 4,870,008; EPO Publ. No. 324 274). Additional leaders employing an alpha-factor leader fragment that provides for secretion include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alpha-factor. (See, e.g., PCT Publ. No. WO 89/02463).

Usually, transcription termination sequences recognized by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes, are well known.

Usually, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a prokaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 (Botstein, *et al.*, *Gene*, *8*: 17-24, 1979), pC1/1 (Brake, *et al.*, *Proc. Natl. Acad. Sci. USA*, *81*: 4642-4646, 1984), and YRp17 (Stinchcomb, *et al.*, *J. Mol. Biol.*, *158*: 157, 1982). In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably

at least about 20. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host (see, e.g., Brake *et al.*, *supra*).

Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome (Orr-Weaver *et al.*, *Methods in Enzymol.*, *101*: 228-245, 1983). An integrating vector may be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector (see Orr-Weaver *et al.*, *supra*). One or more expression constructs may integrate, possibly affecting levels of recombinant protein produced (Rine *et al.*, *Proc. Natl. Acad. Sci. USA*, *80*: 6750, 1983). The chromosomal sequences included in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as ADE2, HIS4, LEU2, TRP1, and ALG7, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418, respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of CUP1 allows yeast to grow in the presence of

copper ions (Butt *et al.*, *Microbiol. Rev.*, *51*: 351, 1987).

Alternatively, some of the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, *inter alia*, the following yeasts: *Candida albicans* (Kurtz *et al.*, *Mol. Cell. Biol.*, *6*: 142, 1986), *Candida maltose* (Kunze *et al.*, *J. Basic Microbiol.*, *25*: 141, 1985), *Hansenula polymorpha* (Gleeson *et al.*, *J. Gen. Microbiol.*, *132*: 3459, 1986; Roggenkamp *et al.*, *Mol. Gen. Genet.*, *202*: 302, 1986), *Kluyveromyces fragilis* (Das *et al.*, *J. Bacteriol.*, *158*: 1165, 1984), *Kluyveromyces lactis* (De Louvencourt *et al.*, *J. Bacteriol.*, *154*: 737, 1983; Van den Berg *et al.*, *Bio/Technology*, *8*: 135, 1990), *Pichia guillerimondii* (Kunze *et al.*, *J. Basic Microbiol.*, *25*: 141, 1985), *Pichia pastoris* (Cregg *et al.*, *Mol. Cell. Biol.*, *5*: 3376, 1985; U.S. Patent Nos. 4,837,148 and 4,929,555), *Saccharomyces cerevisiae* (Hinnen *et al.*, *Proc. Natl. Acad. Sci. USA*, *75*: 1929, 1978; Ito *et al.*, *J. Bacteriol.*, *153*: 163, 1983), *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, *300*: 706, 1981), and *Yarrowia lipolytica* (Davidow *et al.*, *Curr. Genet.*, *10*: 39, 1985; Gaillardin *et al.*, *Curr. Genet.*, *10*: 49, 1985).

Methods of introducing exogenous DNA into yeast hosts are well-known in the art, and usually include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformation procedures usually vary with the yeast species to be transformed (see, e.g., Kurtz *et al.*, *Mol. Cell. Biol.*, *6*: 142, 1986; Kunze *et al.*, *J. Basic Microbiol.*, *25*: 141, 1985 [*Candida*], Gleeson

et al., *J. Gen. Microbiol.*, *132*: 3459, 1986; Roggenkamp *et al.*, *Mol. Gen. Genet.*, *202*: 302, 1986 [*Hansenula*], Das *et al.*, *J. Bacteriol.*, *158*: 1165, 1984; De Louvencourt *et al.*, *J. Bacteriol.*, *154*: 737, 1983; Van den Berg *et al.*, *Bio/Technology*, *8*: 135, 1990 [*Kluyveromyces*], Cregg *et al.*, *Mol. Cell. Biol.*, *5*: 3376, 1985; Kunze *et al.*, *J. Basic Microbiol.*, *25*: 141, 1985; U.S. Patent Nos. 4,837,148 and 4,929,555 [*Pichia*], Hinnen *et al.*, *Proc. Natl. Acad. Sci. USA*, *75*: 1929, 1978; Ito *et al.*, *J. Bacteriol.*, *153*: 163, 1983 [*Saccharomyces*], Beach and Nurse, *Nature*, *300*: 706, 1981 [*Schizosaccharomyces*], and Davidow *et al.*, *Curr. Genet.*, *10*: 39, 1985; Gaillardin *et al.*, *Curr. Genet.*, *10*: 49, 1985 [*Yarrowia*]).

Toxins are molecules which adhere to the surface of cells by binding to specific cell receptors. After binding, the toxins enter the cells and exert their toxic function. Numerous toxins have so far been discovered and many of them have been characterised in detail at the biochemical and genetic level (see, for example, Alouf and Freer, 1991).

ADP-ribosylation is a post-translational modification performed by viral, bacterial and eukaryotic enzymes. The present knowledge of these enzymes derives mainly from biochemical, genetic and three-dimensional studies on various bacterial toxins produced by *Pseudomonas aeruginosa* (PAETA), *Corynebacterium diphtheriae* (DT), *Bordetella pertussis* (PT), *Vibrio cholerae* (CT), and *E. coli* (LT) (Domenighini and Rappuoli, 1996). It was in these bacteria that the ADP-ribosylation enzymatic reaction was first discovered. To date, in addition to the above toxins, the amino acid sequences of more than 30 enzymes with ADP-ribosylating activity are known, including all eukaryotic mono-ADP-ribosyltransferases (MARTs) and poly-ADP-ribosylpolymerases (PARPs), bacterial ADP-ribosylating enzymes which do not have toxic activity, and the analogous enzymes encoded by T-even bacteriophages (Domenighini and

Rappuoli, 1996).

Consequently, in the context of the present invention, toxin-like molecules are those molecules which possess similar properties to toxins. Preferred examples of toxin-like molecules include ADP-ribosylating enzymes such as eukaryotic MARTs, T-cell alloantigens such as RT6, RT6-1 and RT6-2, PARPs, the bacterial metabolism regulation factor, DraT, or enzymes encoded by bacteriophages such as T2, T4 and T6 (Domenighini and Rappuoli, 1996).

In many toxins, the regions and/or the amino acid residues responsible for the toxic activity have been identified and it has been shown that modification, deletion or substitution of such regions and/or residues make toxins unable or less able to exert their activity. In many cases such detoxified toxins are still able to bind and enter cells. This property has been exploited to deliver molecules to specific cells and tissues and important medical applications have been envisaged (see, for example, Pugsley, 1996).

The present invention covers the novel use of toxins (and toxin-like molecules) as vehicles to deliver DNA into cells. Some of the advantages of using such molecules to deliver DNA into cells over systems of DNA delivery currently in use are: (1) efficiency (a large number of toxin receptors are located on the surface of the cells); (2) specific delivery (the receptors for some toxins are located only on the surface of particular cells and tissues); and (3) built-in immunogenicity and adjuvanticity (many toxins are good immunogens and adjuvants - such properties might be particularly useful when the methods described herein are used for DNA immunization).

The present invention shall now be described by way of the following examples which show how toxins can be engineered to deliver DNA to cells. The use of appropriately

engineered *E. coli* enterotoxin (LT), lethal factor (LF) of anthrax toxin and diphtheria toxin (DT) of *C. diphtheriae* is described. However, as would be clear to one skilled in the art, other toxins or toxin-like molecules could also be used.

The present invention is illustrated in the appended examples, with reference to the following figures:

Figure 1 shows the construction of plasmid pT7/LTbpH1 through the cloning of the LT/bpH1 hybrid gene into the vector pT7-7. The gene coding for the A and B subunits of LT of *E. coli*, where the region coding for the A1 domain carrying the toxic activity of LT was replaced with a 165 bp fragment of the bpH1 sequence of *B. pertussis*, was cloned into pT7-7 to express the LT/BpH1 fusion protein.

Figure 2 shows a South-Western analysis of radiolabelled DNA probe binding to LT/BpH1 fusion protein. Bacterial total protein extracts were fractionated on a 15% SDS-acrylamide gel and blotted onto a nitrocellulose filter. The filter was hybridised with pGEM®-3 radiolabelled DNA.
Lane 1: *B. pertussis* total protein extract;
Lane 2: pT7/LTbpH1-transformed *E. coli* total protein extract after induction of expression of the hybrid gene;
Lane 3: pT7/LTbpH1-transformed *E. coli* total protein extract without induction of expression of the hybrid gene.

Figure 3 shows plasmid pGST/LF_NbpH1, which was constructed through the cloning of LF_N and bpH1 into the pGEX-KG vector. LF_N sequence coding for amino acid residues 1-254 and the bpH1 sequence coding for amino acid residues 1-55 were cloned into the pGEX-KG vector to express a GST/LF_NBpH1 fusion protein; the GST protein is then cleaved by thrombin digestion.

Figure 4 shows a South-Western analysis of radiolabelled DNA probe binding to GST/LF_NBpH1 fusion protein. Bacterial

total protein extracts were fractionated on a 12% SDS-acrylamide gel and blotted onto a nitrocellulose filter. The filter was hybridised with pGEM®-3 radiolabelled DNA.
Lane 1: pGST/LF_NbpH1-transformed *E. coli* total protein extract without induction of expression of the hybrid gene;
Lane 2: pGST/LF_NbpH1-transformed *E. coli* total protein extract after induction of expression of the hybrid gene;
Lane 3: *B. pertussis* total protein extract.

Figure 5 shows the *gal/dtb* hybrid gene as cloned in plasmid pTrcHis-GAL/DTB. Plasmid pTrcHis-GAL/DTB was constructed through the cloning of the *gal/dtb* hybrid gene into the pTrcHis A vector. *GAL4* sequence, coding for amino acid residues 2-147, and *tox197* sequence, coding for amino acid residues 195-560, were cloned into the pTrcHis A vector to express the GAL/DTB fusion protein. Nucleotides between restriction sites *Sac*I and *Kpn*I belong to pGEM®-3 sequence.

Figure 6 shows a South-Western analysis of radiolabelled DNA probe binding to GAL/DTB fusion protein.
Lane 1: 2 µg of DT protein;
Lane 2: 9 µg of GAL/DTB fusion protein before dialysis;
Lane 3: 2 µg of GAL/DTB fusion protein after dialysis in 500 mM L-arginine, 50 mM phosphate buffer pH 7.0, 0.5 mM glutathione (oxidated), 5 mM glutathione (reduced), 10% glycerol.

Figure 7 shows a Fluorescence Activated Cell Sorter (FACS) analysis of the binding of the GAL/DTB fusion protein to Vero cells.

Figure 8 shows a FACS analysis of the internalisation of the GAL/DTB fusion protein into Vero cells.

EXAMPLES

EXAMPLE I

Engineering LT to bind DNA

The strategy used to modify LT is described in Figure 1. The gene encoding the A and B subunits of LT of *E. coli* was isolated from the plasmid pBS/LT (Pizza *et al.*, 1994) and the region coding for the A1 domain, carrying the toxic activity, was replaced with a 165 bp DNA fragment isolated by PCR from *Bordetella pertussis* chromosomal DNA. The protein encoded by the amplified fragment has been described as a double-stranded DNA-binding protein homologous to a histone H1 (Scarlato *et al.*, 1995) and named BpH1. The hybrid gene was inserted into the expression vector pT7-7 (Tabor and Richardson, 1985) and the plasmid (pT7/LTbpH1) was used to transform *E.coli* cells. The recombinant clones obtained were able to express a fused product (detectable by Western blot) which was able to bind DNA as judged by South-Western analysis (Figure 2).

Experimental approach

(1) Isolation of the LT-A subunit DNA

A 580 bp gene fragment encoding the first 28 amino acids of the A1 subunit of LT was derived by *Sma*I/*Xba*I digestion of plasmid pBS/LT.

(2) Isolation of a DNA fragment from the bpH1 gene

The bpH1 165 bp DNA region coding for amino acids 1-55 was amplified by polymerase chain reaction (PCR) using the following synthetic oligodeoxynucleotides:

(i) 5'-tctagaATGGCAACTGCCAAGAAGGCC; and

(ii) 5'-aagcttGCTTCTTCGCGGCGACCTTCTTC;

where the *Xba*I and *Hin*dIII restriction sites (underlined lower case letters) were introduced at the 5' and 3' ends, respectively.

PCR was performed on 200 ng of plasmid pG23.1EB as template containing the 901 bp *Eco*RI-*Bam*HI fragment of bpH1 (Scarlato *et al.*, 1995). The reaction was performed in a volume of 100 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 2% DMSO, 50 pmole of each primer, 200 µM of each dNTP, 2.5 U *Taq* DNA polymerase (Perkin Elmer, USA). DNA was denaturated for 5 min at 97°C and PCR performed for 30 cycles: 30 sec at 97°C, 20 sec at 64°C, 20 sec at 72°C. The amplified fragment was digested with *Xba*I and *Hin*dIII restriction enzymes and gel-purified.

(3) Construction of the LT-A/bpH1 hybrid gene

The 580 bp *Sma*I/*Xba*I LT fragment, derived from plasmid pBS/LT, was cloned into the *Sma*I/*Xba*I sites of pEMBL18 vector (Dente *et al.*, 1983). Subsequently the 165 bp bpH1 amplified region was cloned 3' of the LT-A1 gene fragment into the *Xba*I/*Hin*dIII sites for the construction of the LT-A1/bpH1 hybrid sequence. The 210 bp sequence coding for the A2 domain of LT was amplified by PCR using the pBS/LT plasmid DNA as template. The PCR reaction was performed using the following synthetic oligodeoxynucleotides:

(i) 5'-AAGCTTGGAGAGAAGAA; and

(ii) 5'-aagcttTCATAATTCATCCCGAATTCTGT;

where the *Hin*dIII restriction site (underlined lower case letters) was introduced at the 3' end.

PCR was performed on 200 ng of plasmid pBS/LT as template

containing the 1840 bp *Sma*I-*Hin*dIII fragment of LT. The reaction was performed in a volume of 100 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 50 pmole of each primer, 200 µM of each dNTP, 2.5 U *Taq* DNA polymerase (Perkin Elmer, USA). DNA was denaturated for 5 min at 94°C and PCR performed for 30 cycles: 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C. The amplified fragment was digested with *Hin*dIII restriction enzyme and gel-purified. The 210 bp LT-A2 amplified region was then cloned 3' of the LT-A1/bpH1 hybrid gene fragment into the *Hin*dIII restriction site for the construction of the LT-A/bpH1 hybrid sequence. Several positive clones were obtained, as determined by restriction enzyme analysis, and the plasmid isolated from one positive clone was named pEM/LTAbpH1. The sequence of the plasmid was analyzed by automatic sequencing.

(4) Cloning of the LT/bpH1 hybrid gene into an expression vector

For bacterial expression of the fusion protein we used plasmid pT7-7 (Tabor and Richardson, 1985) that contains a T7 promoter and is used to express genes using T7 RNA polymerase. A 460 bp DNA fragment encoding the LT-A/BpH1 fused protein was amplified by PCR using the DNA of plasmid pEM/LTAbpH1 as template. The fragment obtained lacked the LT constitutive promoter region. The PCR reaction was performed using the following synthetic oligodeoxynucleotides:

(i) 5'-catatgAAAAATATAACTTTCATTTTTTTTATT; and

(ii) 5'-AAGCTTTCATAATTCATCCCGAATTCTGT;

where the *Nde*I restriction site (underlined lower case letters) was introduced at the 5' end of the DNA fragment.

PCR was performed on 20 ng of plasmid pEM/LTAbpH1 as

template. The reaction was performed in a volume of 100 µl containing 10 mM Tris-HCl pH 8.8, 10 mM KCl, 0.002% v/v Tween 20, 1 mM $MgCl_2$, 50 pmole of each primer, 40 µM of each dNTP, 3 U UlTma DNA polymerase (Perkin Elmer, USA). DNA was denaturated for 2 min at 94°C and PCR performed for 30 cycles: 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C. The amplified fragment was digested with *NdeI/EcoRI* restriction enzymes and gel-purified.

The DNA fragment coding for the B domain of the LT toxin was isolated from plasmid pBS/LT by *EcoRI/HindIII* restriction enzyme digest. The two DNA fragments coding for the LT-A/BpH1 and the LT-B domains of the fused protein were then cloned into the *NdeI/HindIII* sites of the pT7-7 vector thereby obtaining pT7/LTbpH1 clones. The plasmid DNA was transformed into the *E. coli* BL21 (DE3) strain carrying the T7 RNA polymerase of bacteriophage λ DE3 integrated into the chromosome (Studier and Moffatt, 1986).

(5) Expression of the fused protein and analysis of its DNA binding capacity

E. coli BL21 cells carrying plasmid pT7/LTbpH1 were grown at 37°C in Luria Bertani (LB) medium containing 50 µg/ml of ampicillin to A_{600nm} of 0.6. Protein expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and incubation was continued for an additional two hours. Cells were harvested by centrifugation, the cell pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 25% saccharose.

Specific DNA-binding activity of the fusion protein (LT/BpH1) derived from pT7/LTbpH1 containing the BpH1 binding protein domain was determined in a South-Western assay. 20 µl of the bacterial suspension was subjected to electrophoresis in a 15% gel of acrylamide-containing SDS and blotted onto a nitrocellulose filter. The filter was saturated in 3% dried milk in PBS for 30 min at room temperature and rinsed with TE Buffer. The filter was

hybridized using as a probe pGEM®-3 (Promega Corporation, Madison, WI, USA) DNA, linearised at the *Eco*RI site. 100 ng of pGEM®-3 DNA was uniformly labelled with α^{32}P dATP and dCTP using a random priming kit (Amersham, UK). Protein-DNA complexes were allowed to form for 20 min at room temperature in TE buffer, rinsed twice in TE buffer, and subjected to autoradiography (Figure 2).

EXAMPLE II

Engineering LF to bind DNA

The strategy used to modify the lethal factor (LF) of anthrax toxin is described in Figure 3. Residues 1-254 of LF (LF_N) have been shown to constitute a functional domain able to bind to PA_{63} and be translocated to the cytosol of the cells, carrying with it certain fused polypeptides (Arora and Leppla, 1993).

PA_{63} is the carboxyl terminal 63 kDa domain of the PA protein that binds to a specific cell surface receptor (Escuyer and Collier, 1991). Cell-bound PA_{63} has been found to expose a site that can bind LF_N, thereby forming an LF_N/PA complex. The LF_N/PA complex is then able to be internalised by receptor-mediated endocytosis and delivered to the endosome, where the low pH environment triggers translocation of the LF_N to the cytoplasm.

The gene sequence encoding the amino terminal domain of LF (LF_N: residues 1-254) of the anthrax toxin of *Bacillus anthracis* was isolated from plasmid pLF7 (Robertson and Leppla, 1986). A 165 bp DNA fragment isolated by PCR from the *Bordetella pertussis* chromosomal DNA, coding for BpH1 (Scarlato *et al.*, 1995), was cloned at the 3' end of the LF_N gene sequence. The hybrid gene was inserted into the expression vector pGEX (Smith and Johnson, 1988) and the plasmid was used to transform *E.coli* cells. The recombinant

clones obtained were able to express a fused product which was able to bind DNA as judged by South-Western analysis (Figure 4).

Experimental approach

(1) Isolation of the LF_N DNA and cloning in pGEX vector

The LF_N sequence was amplified from plasmid pLF7 (Robertson and Leppla, 1986) using the same primers as described by Arora *et al.* (1994), where the *Xba*I restriction site was inserted at the 5' end of the LF_N fragment and the *Nco*I site was added at the 3' end of the fragment. The LF_N PCR product was digested sequentially with *Xba*I and *Nco*I restriction enzymes. The digested PCR product was then cloned into the pGEX-KG vector (Smith and Johnson, 1988) that had been previously digested with the same enzymes.

The vector pGEX-KG is designed for inducible high level intracellular expression of a gene or gene fragment of interest as a fusion protein with *Schistosoma japonicum* Glutathione S-transferase (GST). A thrombin cleavage site is downstream from the multiple cloning site. The vector is derived from pGEX-2T (Pharmacia Biotech, Inc., Uppsala, Sweden) and has the coding sequence for five glycines inserted between the thrombin cleavage site and the multiple cloning site (Guan and Dixon, 1991). The gene expression is controlled by the *tac* promoter, which is induced using the lactose analogue isopropyl-β-D-thiogalactoside (IPTG).

(2) Isolation of a DNA fragment from the bpH1 gene

The bpH1 165 bp DNA region coding for amino acids 1-55 was amplified by polymerase chain reaction (PCR) using the following synthetic oligodeoxynucleotides:

(i) 5'-ccatggCAACTGCCAAGAAGGCC; and

(ii) 5'-ctcgagTCACTTCTTCGCGGCGACCTTCTT;

where the *Nco*I and *Xho*I restriction sites (underlined lower case letters) were introduced at the 5' and 3' ends, respectively.

PCR was performed on 100 ng of plasmid pG23.1EB as template containing the 901 bp *Eco*RI-*Bam*HI fragment of bpH1. The reaction was performed in a volume of 100 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 2% DMSO, 50 pmole of each primer, 200 µM of each dNTP, 2.5 U *Taq* DNA polymerase (Perkin Elmer, USA). DNA was denaturated for 5 min at 97°C and PCR performed for 30 cycles: 30 sec at 97°C, 30 sec at 64°C, 30 sec at 72°C. The amplified fragment was digested with *Nco*I and *Xho*I restriction enzymes and gel-purified.

(3) Cloning of the LF_N/bpH1 hybrid gene in an expression vector

The 165 bp bpH1 amplified region was cloned 3' of the GST-LF_N gene into the *Nco*I/*Xho*I sites of the pGEX expression vector for the construction of the GST-LF/bpH1 hybrid sequence. Several positive clones were obtained, as determined by restriction enzyme analysis, and named pGST/LF_NbpH1. The sequences of 3 positive clones were analyzed by automatic sequencing. The plasmid DNA of the 3 positive clones (clones 4, 10 and 17) was transformed into the *E. coli* expression host TG1 (Gibson, 1984).

(4) Expression of the fused protein and analysis of its DNA binding capacity

E. coli TG1 cells carrying plasmid pGST/LF_NbpH1 were grown at 37°C in LB medium containing 50 µg/ml of ampicillin to A_{600nm} of 1.0. Protein expression was induced by addition of 0.2 mM isopropyl-β-D-thiogalactoside and incubation was continued for an additional three hours. Cells were

harvested by centrifugation, the cell pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 25% saccharose. Cell lysates were then prepared by mild sonication on ice and cleared by centrifugation. Samples were analyzed for the expression of the fusion protein by SDS-PAGE analysis. 20 µl of the cell sonicates were loaded onto a 12% SDS-polyacrylamide gel. The gel was stained with Coomassie Blue to visualise the parental pGST-LF protein and the fusion product GST/LF_NBpH1.

DNA-binding activity of the fusion protein containing the bpH1-binding protein domain was determined in a South-Western assay. 20 µl of the bacterial suspension was subjected to electrophoresis in a 12% SDS-polyacrylamide gel and blotted onto a nitrocellulose filter. The filter was saturated in 3% dried milk in PBS for 30 min at room temperature and rinsed with TE buffer. The filter was hybridized using as a probe pGEM®-3 (Promega Corporation, Madison, WI, USA) DNA, linearised at the *Eco*RI site. 100 ng of pGEM®-3 DNA was uniformly labelled with α^{32}P dATP and dCTP using a random priming kit (Amersham, UK). Protein-DNA complexes were allowed to form for 20 min at room temperature in TE buffer, rinsed twice in TE buffer, and subjected to autoradiography (Figure 4).

EXAMPLE III

<u>Transfection of toxin-sensitive cells with DNA bound to fusion products LT/BpH1 and LF_N/BpH1</u>

The fusion proteins LT/BpH1 (Example I) and LF_N/BpH1 (Example II), containing detoxified toxin residues, can be incubated with an appropriate amount of transfecting DNA in order to bind the DNA to the BpH1 residues of the fusion proteins.

The transfecting DNA can essentially be any double-stranded DNA capable of transfecting (through current methods of

transfection) the toxin-sensitive cells. A suitable DNA would be a plasmid containing a selection marker for eukaryotic cells that would allow selection in culture of positively transfected cells. Alternatively, a plasmid containing the gene sequence of the Green Fluorescence Protein (GFP) (Clontech, Palo Alto, CA, USA) would allow selection of positive cells by monitoring the expression of GFP in cells by flow cytometry. Furthermore, a plasmid containing the Luciferase reporter gene (Promega Corporation, Madison, WI, USA) would allow the visualisation of positive cells by using a chemiluminescence assay.

EXAMPLE IV

Engineering DT to bind DNA

The gene encoding the CRM197 diphtheria toxin (DT) mutant depleted of its toxic moiety (A domain) was amplified from a recombinant M13 mp8 plasmid carrying a 1.88 kb fragment encoding the *tox* operon (Giannini *et al.*, 1984). The amplification product was fused to a 439 bp fragment isolated by PCR from plasmid pGBT9 (Clontech, Palo Alto, CA, USA). The 439 bp amplified fragment encodes amino acids 2-147 of the yeast protein GAL4, a transcriptional activator which is able to bind to a specific double-strand DNA sequence (Carey *et al.*, 1989). The hybrid gene was inserted into the expression vector pTrcHis A (Invitrogen Corporation, San Diego, CA, USA) and the resulting plasmid pTrcHis-GAL/DTB was used to transform *E. coli* cells. The recombinant clones obtained were able to express a fused protein which specifically recognized the GAL4-specific DNA sequence. The fusion protein, termed GAL/DTB, was also able to bind to DT receptors, as judged by binding analysis on African green monkey Vero cells.

Experimental approach

(1) Isolation of a DNA fragment from the CRM197 protein-encoding gene (*tox197*).

A 1120 bp DNA fragment encoding amino acids 195-560 of CRM197 was isolated by PCR from plasmid M13 mp8 using the following synthetic oligodeoxynucleotides:

(i) 5'- CATCGgtcgacGGAAAACGTGGCCAA; and

(ii) 5'- TCCACaagcttTCAGCTTTTGATTTC;

where the *Sal*I and *Hind*III restriction sites (underlined lower case letters) were introduced at the 5' and 3' ends, respectively.

PCR was performed on 70 ng of plasmid M13 mp8 as template, containing the 1683 bp *Eco*RI/*Hind*III fragment of *tox197*. The reaction was performed in a volume of 100 µl containing 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 2% DMSO, 50 picomoles of each primer, 200 µM of each dNTP and 5 U of *Taq* DNA polymerase (Boehringer Mannheim, Germany). DNA was denatured for 5 min at 94°C and PCR was performed for 30 cycles: 30 sec at 94°C, 30 sec at 42°C, 1 min at 72°C. The amplified fragment was digested with *Sal*I and *Hind*III restriction enzymes and gel-purified.

(2) Isolation of a DNA fragment from the *GAL4* gene

The *GAL4* 439 bp DNA fragment encoding amino acids 2-147 was amplified by PCR from plasmid pGBT9 (Clontech, Palo Alto, CA, USA) using the following synthetic oligodeoxynucleotides:

(i) 5'- ACGAAggtaccATGAAGCTAACTGTCT; and

(ii) 5'- CAACCgtcgacTACAGTCAACTGTCT;

where the *Kpn*I and *Sal*I restriction sites (underlined in lower case letters) were introduced at the 5' and 3' ends, respectively.

PCR was performed on 50 ng of plasmid pGBT9 as template, containing the 460 bp *Hin*dIII/*Eco*RI fragment of the *GAL4* gene. The reaction was performed in a volume of 100 µl containing 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 2% DMSO, 50 picomoles of each primer, 200 µM of each dNTP and 5 U of *Taq* DNA polymerase (Boehringer Mannheim, Germany). DNA was denatured for 5 min at 94°C and PCR was performed for 30 cycles: 30 sec at 94°C, 30 sec at 42°C, 1 min at 72°C. The amplified fragment was digested with *Kpn*I and *Sal*I restriction enzymes and gel-purified.

(3) Construction of the *gal/dtb* hybrid gene

The 439 bp *Kpn*I/*Sal*I fragment of the *GAL4* gene obtained by PCR was cloned into *Kpn*I/*Sal*I sites of pGEM®-3 cloning vector (Promega Corporation, Madison, WI, USA). Subsequently, the 1120 bp *tox197* amplified region was cloned 3' of the *GAL4* gene fragment into the *Sal*I/*Hin*dIII sites for the construction of the GAL/DTB-encoding hybrid sequence, giving rise to plasmid pGEM-GAL/DTB.

(4) Cloning of the *gal/dtb* hybrid gene into an expression vector

For bacterial expression of the fusion protein GAL/DTB the plasmid pTrcHis A (Invitrogen Corporation, San Diego, CA, USA) was used. This plasmid contains a Trc promoter and a histidine tag to easily purify the resulting protein. The DNA fragment coding for the GAL/DTB fusion protein was isolated from plasmid pGEM-GAL/DTB by *Sac*I/*Hin*dIII restriction enzyme digestion (note that nucleotides between *Sac*I and *Kpn*I restriction sites of the *Sac*I/*Hin*dIII fragment belong to the pGEM®-3 sequence). The *Sac*I/*Hin*dIII fragment

derived from plasmid pGEM-GAL/DTB was cloned into the *Sac*I/*Hin*dIII sites of pTrcHis A (Figure 5), giving rise to the expression plasmid pTrcHis-GAL/DTB.

(5) Expression and purification of GAL/DTB protein

E. coli DH5α cells (Hanahan, 1983) carrying plasmid pTrcHis-GAL/DTB were grown at 37°C in 1.7 l of Luria-Bertani (LB) medium containing 100 μg/ml ampicillin to A_{590nm} of 0.7. Protein expression was induced by addition of 1 mM IPTG and incubation was continued for an additional 4 hrs. Cells were harvested by centrifugation and resuspended by stirring for 1 hr at room temperature in 15 ml of 6 M guanidine-HCl, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 8 (buffer A). Subsequently, the mix was added to 4 ml of nickel-conjugated agarose (Qiagen GmbH, Germany) equilibrated in buffer A and, after 30 min stirring at room temperature, it was loaded on a 1.6 cm diameter column. The column was washed with 10 volumes of buffer A and 5 volumes of 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 8. Additional washing with 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 6 were performed until $O.D._{280nm}$ of the eluate was <0.01. Elution of the GAL/DTB fused protein was achieved by a linear gradient of pH (from 6.5 to 4) in 60 ml of 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl; the fused protein eluted at a pH between 4 and 4.5.

To remove the urea, fractions containing GAL/DTB protein were pooled and dialysed overnight at room temperature against 4 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 7, 5% glycerol. The resulting GAL/DTB solution was again dialysed overnight at 4°C against 2 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl pH 7, 10% glycerol. For complete renaturation, the 2 M urea solution of GAL/DTB protein was diluted to 100 μg/ml in 500 mM L-arginine, 50 mM phosphate buffer pH 7, 0.5 mM glutathione (oxidated), 5 mM glutathione (reduced), 10% glycerol and dialysed overnight at 4°C against the same buffer.

(6) Analysis of DNA-binding capacity of GAL/DTB protein

Specific DNA-binding activity of the fusion protein was determined in a South-Western assay. 20 µl of purified GAL/DTB protein (100 ng/µl) were subjected to electrophoresis in a 15% SDS-polyacrylamide gel and blotted onto a nitrocellulose filter. The filter was saturated in 3% dried milk, 0.1% Triton X-100 in PBS for 30 min at room temperature and rinsed in binding buffer (50 mM Hepes pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 10 µM $ZnSO_4$, 6% glycerol, 200 µg/ml bovine serum albumin (BSA)).

As a probe, the following two synthetic complementary oligodeoxynucleotides, containing two consecutive binding sites for GAL4 protein, were used:

(i) 5'- CGCGTGGATCCGGAGGACAGTCCTCCGGAGACCGGAGGACAGTCCTCCG; and

(ii) 5'- GATCCGGAGGACTGTCCTCCGGTCTCCGGAGGACTGTCCTCCGGATCCA.

1.5 pmoles of the annealed oligos were labelled by a "fill-in" reaction using α^{32}P dATP and dCTP and the Klenow fragment of DNA polymerase. Protein-DNA complexes were allowed to form for 30 min at room temperature in binding buffer, rinsed twice in binding buffer and subjected to autoradiography (Figure 6).

As can be seen in Figure 6, only the GAL/DTB fusion protein was able to bind the radiolabelled probe DNA (lanes 2 and 3), whereas purified DT did not (lane 1).

(7) Analysis of GAL/DTB binding to Vero cells

African green monkey kidney Vero cells were maintained in D-MEM medium supplemented with 5% heat-inactivated foetal calf serum (FCS) at 37°C in a humidified atmosphere of air

and 5% CO_2. For the binding assay, cells were recovered, resuspended in PBS containing 5% FCS (PBS-5% FCS) and seeded on a 96-well tissue culture plate at a density of 10^5 cells/well in a volume of 10 µl. Increasing amounts of GAL/DTB protein (final concentration ranging from 0.006 µg/ml to 54 µg/ml) were added to each well in a final reaction volume of 20 µl. GAL/DTB-cells complexes were incubated overnight at 4°C, rinsed twice in PBS-5% FCS, resuspended in 10 µl of PBS-5% FCS and stained for 45 min on ice with a polyclonal anti-GAL4 (1-147) antibody raised in rabbit (Santa Cruz Biotechnology, CA, USA) at a final concentration of 1 µg/ml in each well. Cells were rinsed twice with PBS-5% FCS, resuspended in 10 µl of PBS-5% FCS and stained for 30 min on ice with anti-rabbit FITC-conjugated antibody (Boehringer Mannheim, Germany) at a final concentration of 12.5 µg/ml in each well. After a final washing with PBS-5% FCS, the cells were resuspended in 200 µl of PBS-5% FCS and subjected to FACS analysis (Figure 7).

As can be seen in Figure 7, GAL/DTB binds Vero cells in a suitable manner as determined by fluorescence intensity measurement. The minimal saturating concentration was in the region of 2 µg/ml.

(8) Analysis of GAL/DTB internalisation into Vero cells

African green monkey kidney Vero cells were maintained in D-MEM medium supplemented with 5% heat-inactivated foetal calf serum (FCS) at 37°C in a humidified atmosphere of air and 5% CO_2. For the binding assay, cells were recovered, resuspended in PBS-5% FCS and seeded on a 96-well tissue culture plate at a density of 10^5 cells/well in a volume of 10 µl. GAL/DTB protein was added to each well at a concentration of 1.5 µg/ml in a final reaction volume of 20 µl. GAL/DTB-cells complexes were incubated overnight at 4°C and then incubated at 37°C for 0, 5, 10, 30 or 60 min. After two rinsing steps in PBS-5% FCS, the cells were

resuspended in 10 µl of PBS-5% FCS and stained for 45 min on ice with a polyclonal anti-GAL4 (1-147) antibody raised in rabbit (Santa Cruz Biotechnology, CA, USA) at a final concentration of 1 µg/ml in each well. Cells were rinsed twice with PBS-5% FCS, resuspended in 10 µl of PBS-5% FCS and stained for 30 min on ice with anti-rabbit FITC-conjugated antibody (Boehringer Mannheim, Germany) at a final concentration of 12.5 µg/ml in each well. After a final washing with PBS-5% FCS, the cells were resuspended in 200 µl of PBS-5% FCS and subjected to FACS analysis (Figure 8).

As can be seen in Figure 8, the mean fluorescence intensity value decreases with time, indicating that the hybrid GAL/DTB protein is internalised by Vero cells. The internalisation process appears to be quite rapid, being 60% accomplished within 5 min of the temperature shift.

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CLAIMS

1. A modified toxin molecule or toxin-like molecule comprising:

(i) one or more toxin or toxin-like amino acid sequences capable of binding to appropriate receptors on a cell surface and being internalised into the cell by receptor-mediated endocytosis, and

(ii) one or more DNA-binding motifs;

wherein the toxin or toxin-like amino acid sequences are incapable of effecting toxicity or only effect low residual toxicity.

2. The modified toxin molecule of claim 1, wherein the toxin amino acid sequences are derived from one or more bacterial toxins.

3. The modified toxin molecule of claim 2, wherein the bacterial toxin(s) is/are derived from *Pseudomonas* sp., preferably *Pseudomonas aeruginosa*, *Corynebacterium diphtheriae*, *Bordetella pertussis*, *Vibrio cholerae*, *Clostridium* sp., preferably *Clostridium botulinum*, or *Escherichia coli*.

4. The modified toxin-like molecule of claim 1, wherein the toxin-like amino acid sequences are derived from one or more ADP-ribosylating enzymes.

5. The modified toxin-like molecule of claim 4, wherein the ADP-ribosylating enzyme(s) is/are eukaryotic mono-ADP-ribosyltransferases (MARTs), T-cell alloantigens, poly-ADP-ribosylpolymerases (PARPs), the bacterial metabolism regulation factor, DraT, or enzymes encoded by T-even bacteriophages.

6. The modified toxin-like molecule of claim 5, wherein the T-cell alloantigens are RT6, RT6-1, or RT6-2.

7. The modified toxin-like molecule of claim 5, wherein the T-even bacteriophages are T2, T4, or T6.

8. The modified toxin or toxin-like molecule of any one of claims 1 to 7, wherein the DNA-binding motif(s) comprise(s) all or part of one or more DNA-binding proteins.

9. The modified toxin or toxin-like molecule of claim 8, wherein the DNA-binding protein is:

(i) a protein with limited specificity for DNA sequence; or

(ii) a protein showing DNA sequence specificity which is capable of selectively binding specific nucleotide motifs.

10. The modified toxin or toxin-like molecule of claim 9, wherein:

(i) the protein with limited specificity for DNA sequence is histone, histone-like protein or polylysine; and/or

(ii) the protein showing DNA sequence specificity is the yeast transcriptional activator GAL4, or an activator or repressor of transcription.

11. The modified toxin or toxin-like molecule of any one of claims 8 to 10, wherein the DNA-binding protein is histone, histone-like protein, optionally BpH1, or the yeast transcriptional activator GAL4.

12. The modified toxin or toxin-like molecule of any one of claims 1 to 11 produced chemically or by genetic engineering.

13. The modified toxin or toxin-like molecule of any one of claims 1 to 12, wherein the modified toxin or toxin-like molecule is further modified to make it capable of binding to appropriate receptors other than receptors specific for all or part of the toxin or toxin-like amino acid sequences of the molecule.

14. The modified toxin or toxin-like molecule of any one of claims 1 to 13 further comprising DNA bound to the DNA-binding motif(s).

15. The modified toxin or toxin-like molecule of claim 14, wherein the bound DNA (i) encodes one or more bacterial, viral, fungal or parasitic proteins, cytotoxic agents, cytolytic agents, antigens, antigenic epitopes, antibodies or fragments thereof, or (ii) comprises one or more replacement genes, augmentative genes or additional genes.

16. The modified toxin or toxin-like molecule of claim 15, wherein the antigens or antigenic epitopes encoded by the DNA are antigens or antigenic epitopes of a pathogen.

17. The modified toxin or toxin-like molecule of claim 15 or claim 16, wherein the antigens or antigenic epitopes encoded by the DNA are protective antigens or protective epitopes of a pathogen.

18. A pharmaceutical composition comprising the modified toxin or toxin-like molecule of any one of claims 1 to 17 and a pharmaceutically acceptable excipient.

19. A process for the production of a modified toxin or toxin-like molecule according to any one of claims 1 to 17 comprising modifying the toxin or toxin-like amino acid sequences by:

(i) eliminating or reducing toxicity, if the amino acid sequences are toxic, and

(ii) incorporating one or more DNA-binding motifs into the molecule.

20. The process of claim 19, wherein the modification for eliminating or reducing toxicity includes addition, deletion, or substitution of amino acids.

21. The process of claim 19, wherein the modification for incorporating the DNA-binding motif(s) is addition of all or part of one or more DNA-binding proteins.

22. The process of claim 21, wherein the DNA-binding protein is as defined by any one or more of the features of claims 9 to 11.

23. The process of claim 21 or claim 22, wherein the addition of all or part of one or more DNA-binding proteins is via one or more chemical or genetically engineered linkage moieties.

24. A process for the production of a pharmaceutical composition according to claim 18 comprising:

(i) modifying the toxin or toxin-like amino acid sequences according to the process of any one of claims 19 to 23, and

(ii) combining the modified toxin or toxin-like molecule of step (i) with a pharmaceutically acceptable excipient.

25. A method of DNA transfer into cells comprising:

(i) binding DNA desired to be transferred into cells to the modified toxin or toxin-like molecule of any one of claims 1 to 17 via one or more of the DNA-binding motifs to form a modified toxin/toxin-like-DNA conjugate,

(ii) incubating the modified toxin/toxin-like-DNA conjugate with appropriate receptor-bearing cells, thereby allowing the conjugate to bind to the cells via the receptors,

(iii) selecting or screening for cells which have been transformed or transfected with the DNA, wherein the DNA has been cellularly internalised via receptor-mediated endocytosis.

26. Use of the modified toxin or toxin-like molecule of any one of claims 1 to 17 in the *in vitro* or *in vivo* transfer of DNA into cells.

27. Use according to claim 26, wherein the DNA to be transferred into cells is bound to one or more of the DNA-binding motifs of the modified toxin or toxin-like molecule, resulting in a modified toxin/toxin-like-DNA conjugate which is then internalised into the cells by receptor-mediated endocytosis.

28. Use of the modified toxin or toxin-like molecule of any one of claims 1 to 17 in the *in vitro* or *in vivo* transformation or transfection of cells.

29. Use of the modified toxin or toxin-like molecule of any one of claims 1 to 17 as a pharmaceutical.

30. Use of the modified toxin or toxin-like molecule of any one of claims 1 to 17 or the pharmaceutical composition of claim 18 in the manufacture of a medicament for:

(i) vaccination;

(ii) gene therapy;

(iii) treating or destroying diseased cells.

31. Use according to claim 30, wherein the vaccination can involve activation of the cytotoxic T-cell immune response or humoral immune response.

32. Use according to claim 30, wherein the gene therapy can include the replacement, augmentation, or addition of genes.

33. Use according to claim 30, wherein the diseased cells can include cancer cells, microbially-infected cells, or abnormal cells.

34. A kit for the *in vitro* or *in vivo* transfer of DNA into cells, comprising the modified toxin or toxin-like molecule of any one of claims 1 to 17 or the pharmaceutical composition of claim 18.

35. A kit for the *in vitro* or *in vivo* transformation or transfection of cells, comprising the modified toxin or toxin-like molecule of any one of claims 1 to 17 or the pharmaceutical composition of claim 18.

1/8

FIGURE 1

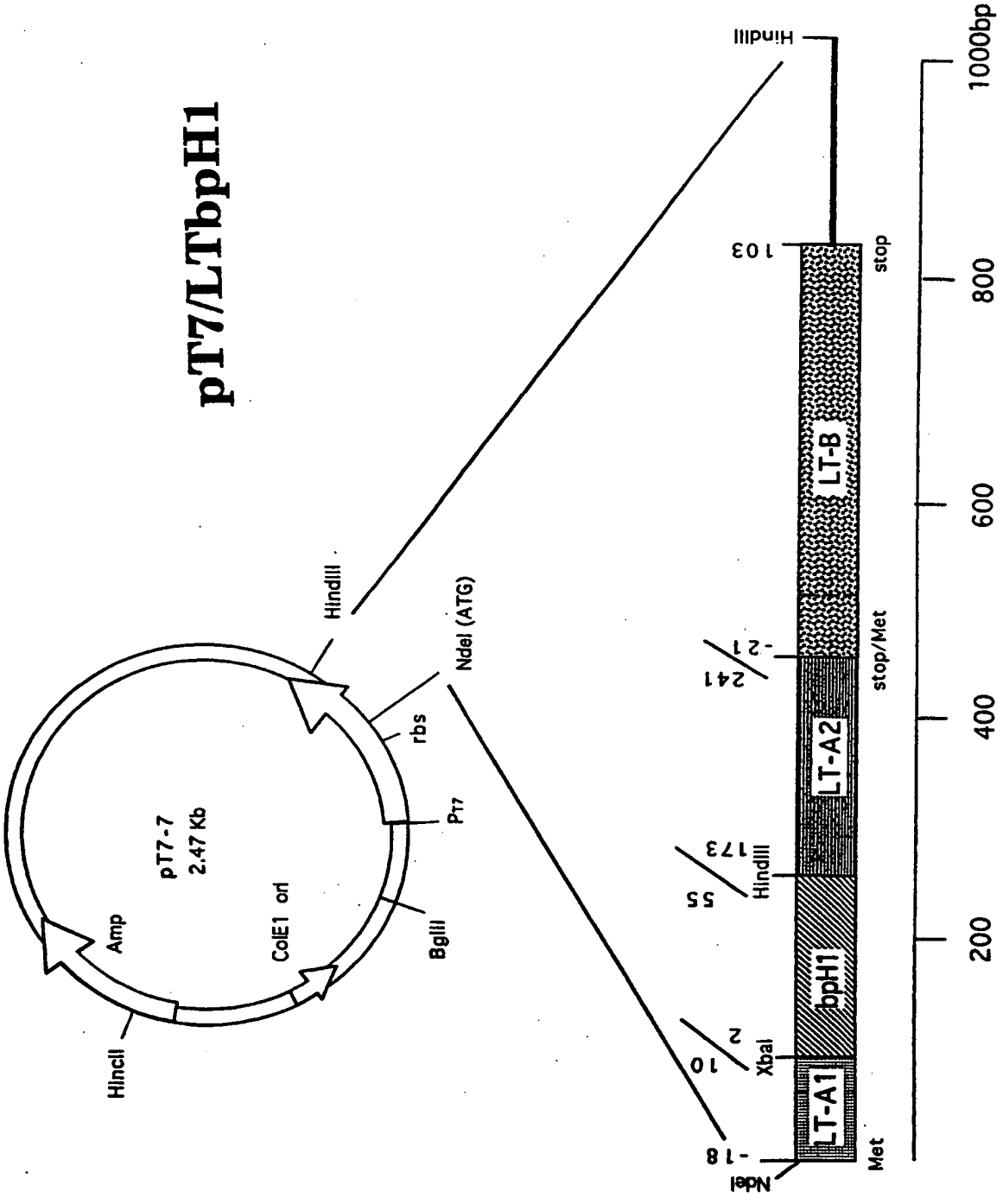

pT7/LTbpH1
pT7-7
2.47 Kb
Amp
ColE1 ori
HincII
BglII
rbs
PT7
HindIII
NdeI (ATG)
HindIII
103
stop
LT-B
-21
241
stop/Met
LT-A2
173
HindIII
55
bpH1
2
XbaI
10
LT-A1
-18
NdeI
Met
200
400
600
800
1000bp

FIGURE 2

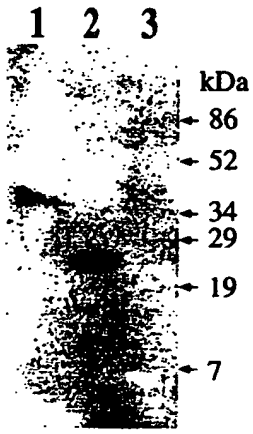


Figure 3

pGST/LFNbpH1

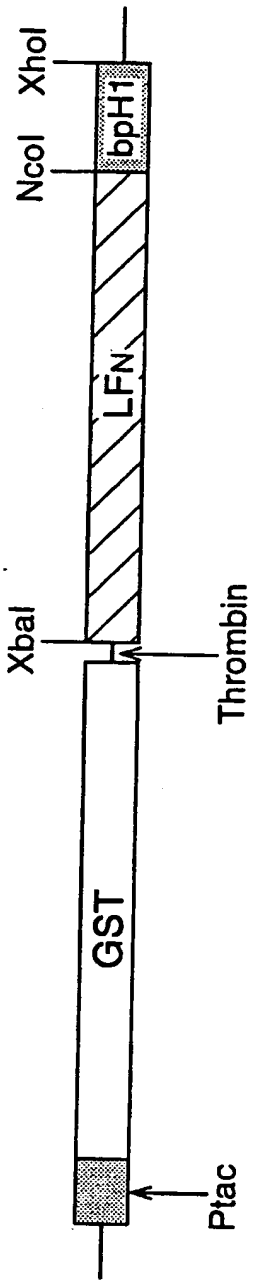


FIGURE 4

Figure 5

***gal/dtb* hybrid gene**

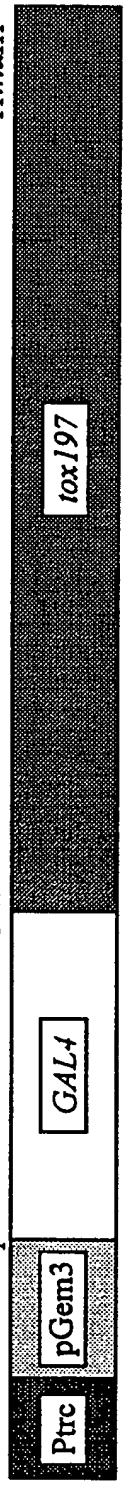


FIGURE 6

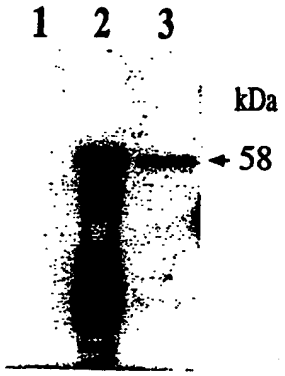


FIGURE 7

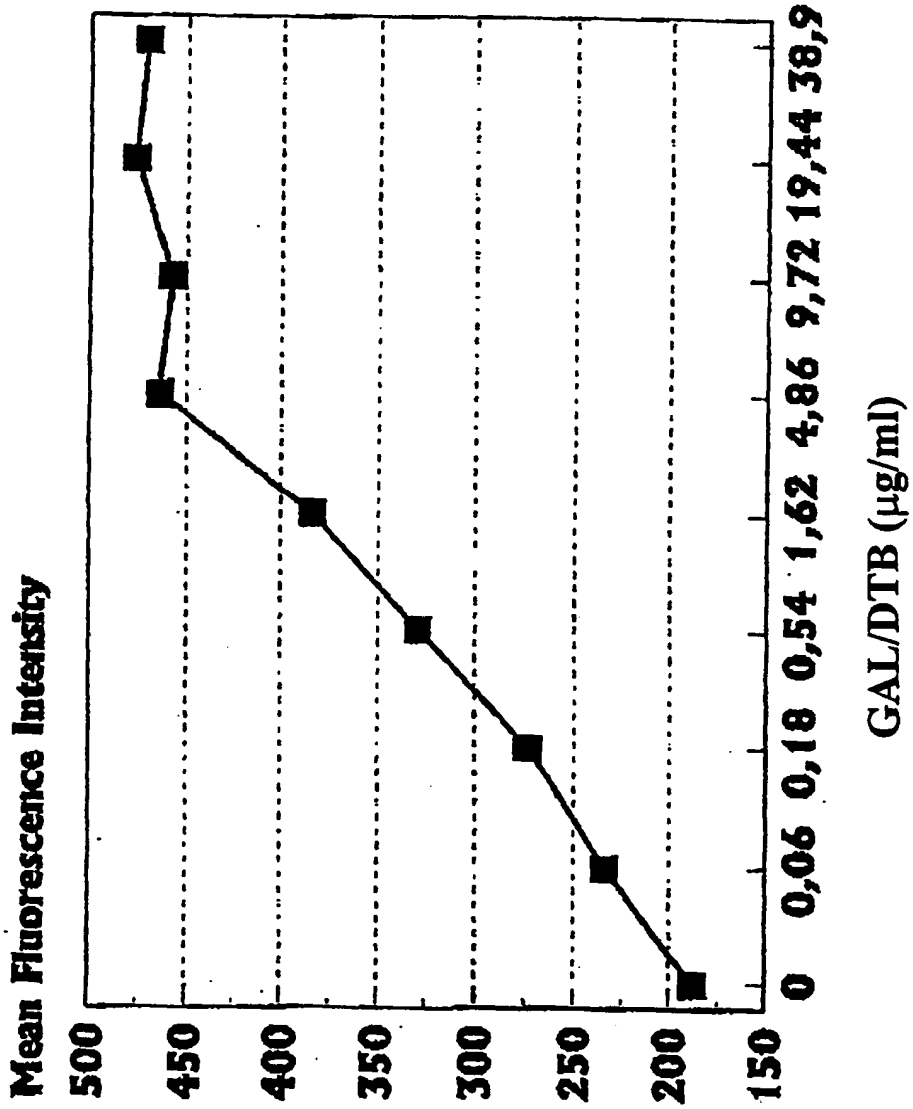
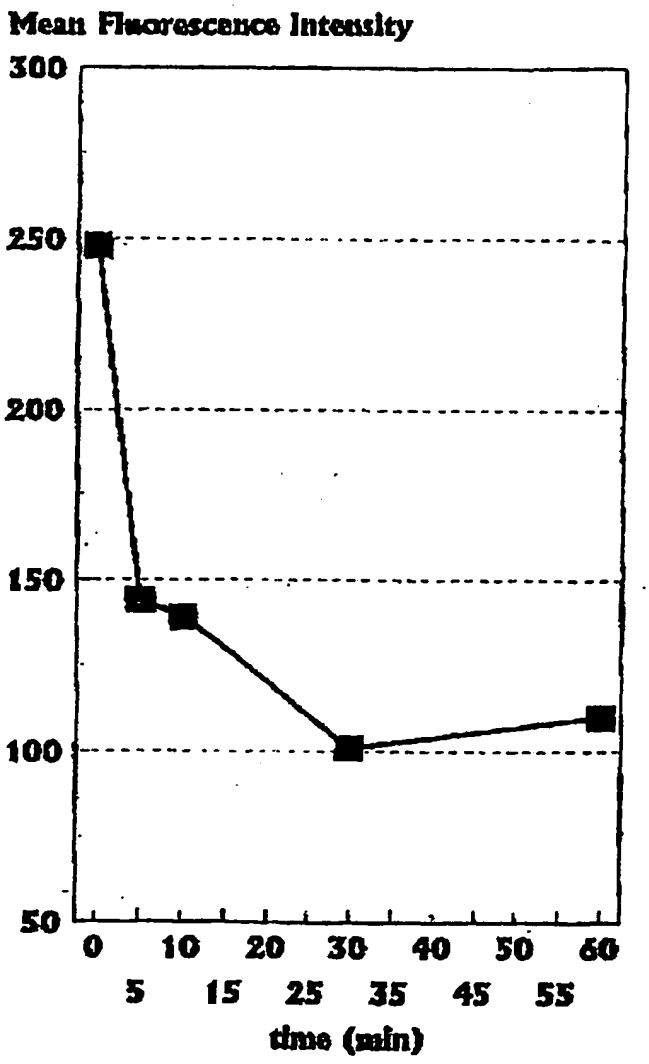

Mean Fluorescence Intensity
500
450
400
350
300
250
200
150
0
0,06
0,18
0,54
1,62
4,86
9,72
19,44
38,9
GAL/DTB (µg/ml)

FIGURE 8


Mean Fluorescence Intensity
300
250
200
150
100
50
0
10
20
30
40
50
60
5
15
25
35
45
55
time (min)

INTERNATIONAL SEARCH REPORT

Int« ional Application No
PCT/IB 98/01005

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C07K14/235 C07K14/245 C07K14/32 C07K14/34
C07K14/395 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 04696 A (MILES INC) 3 March 1994 see page 5, line 15 - line 21 see page 6, line 25 - line 36 see page 7, line 24 - page 8, line 24	1-5,8-35
X	WO 97 05267 A (MAXIM PHARMACEUTICALS) 13 February 1997 see page 3, line 12 - page 5, line 24 see page 11, line 24; claims 1-48; examples 1-7	1-5,8-35
A	WO 95 22618 A (DANA FARBER CANCER INST INC ;CHEN SI YI (US); MARASCO WAYNE A (US)) 24 August 1995 see the whole document	1-35

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure. use. exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents. such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1998	18/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Hornig, H

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IB 98/01005

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 28494 A (TARGETED GENETICS CORP ;OVERELL ROBERT W (US); WEISSER KAREN E (US) 26 October 1995 see page 3, line 13 - page 5, line 12 see page 15, line 14 - page 18, line 16	1-35
A	WO 96 13599 A (WELS WINFRIED ;FOMINAYA JESUS (CH)) 9 May 1996 see page 10, line 18 - line 31; example 2	1-35
P,X	WO 97 23236 A (STARNBACH MICHAEL N ;BALLARD JIMMY D (US); BLANKE STEVEN R (US); C) 3 July 1997 see page 16, line 1 - line 28; claims 1,2,16; figure 4 see page 20, line 1 - line 8	1-5,8-35

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 98/01005

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 26 - 29
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/IB 98/01005

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0789776 A	20-08-1997
		JP 9511916 T	02-12-1997
WO 9723236 A	03-07-1997	AU 2240197 A	17-07-1997
		EP 0866718 A	30-09-1998